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13. ABSTRACT (Maximum 200 Words)

Under this proposal we plan to identify genes that drive the homing of invasive primary breast cancer to its metastatic sites.

For the discovery of such genes, in vivo phage display of breast cancer cDNA libraries will be used. Validation will be achieved by expression monitoring in histological sections as well as by functional assays. In a first series of experiments we established the methodology for in situ hybridization of tissue microarrays containing paraffin-embedded breast cancers for homing genes that were discovered using the liver as a target organ. No distinct expression was found for breast cancer vs. non-cancerous breast tissues.

It is planned to next select homing genes with the breast cancer cell library derived from MDA-MB 231 cells. Then, homing genes derived from that library will be screened against the breast cancer tissue microarray.

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INTRODUCTION

The major problem of clinical cancer is metastasis to distant organs [1-3]. It has been known for over 100 years that there is a close relationship between "seed and soil" in terms of organ-specific metastasis and we now have the tools available to potentially identify tumor cell-derived proteins that aid in the organ-specific metastasis. Here we plan to isolate from human breast cancer cells genes that are candidates for the homing in of tumor cells to blood vessels in distinct organs of metastasis. This will be accomplished using phage display of cDNA libraries and will identify protein fragments that serve as ligands for the local homing in.

In addition to our better understanding of tumor cell metastasis in the body, discoveries made under this proposal will allow us to utilize such genes for both diagnostics and prognostic as well as a therapeutic targeting in the future.

Task 1: Identification of protein fragments that allow for organ-specific metastasis.

Task 2: Study expression patterns of candidate homing genes.

Task 3: Study the functional role of candidate homing genes.

BODY

Work accomplished during the current award cycle:

This work is related to Task 1 and Task 2:

During the current funding cycle, we studied the expression in breast cancer of liver homing genes that were identified using colon cancer-derived liver homing genes. Figure 1 shows an example of an in situ hybridExpression patterns in human breast cancer tissue arrays that contain different stage invasive primary cancers (64 each of invasive breast cancers with and without lymph node and 64 with distant metastases; Details in "Methods".).

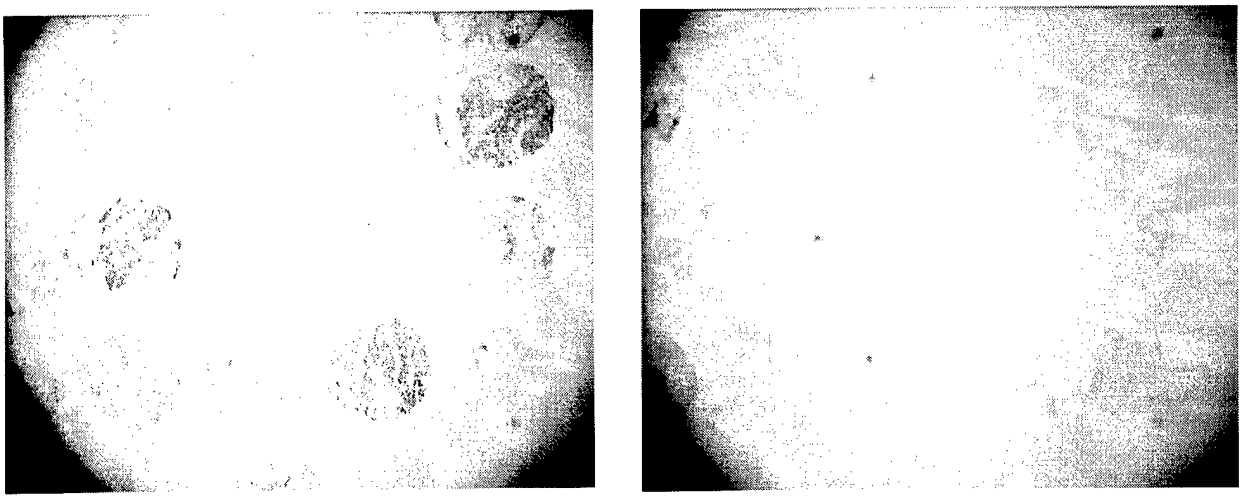

Figure 1. In situ hybridization of the LS42 cDNA to breast cancer samples (left) and normal, non-tumor tissues from different organs (right). The LS42 cDNA is one of the genes isolated as a liver homing gene using metastatic colon cancer cells (LS178T). This is an example to illustrate results from in situ hybridization. Details and other examples are given in the **appendix materials**.

We compared the expression pattern of different liver homing genes in primary breast cancers of different stages relative to non-malignant breast tissue (Figure 2). Overall a relatively low frequency of expression of the liver homing genes was observed in the breast cancers (around 30% for three of the genes and close to 50% for one of them). No significant difference was seen when comparing cancer and normal breast tissues, 20% and 30% frequency (Figure 2). This suggests that breast cancer may not use the homing genes isolated from liver metastasis of colon cancer cells.

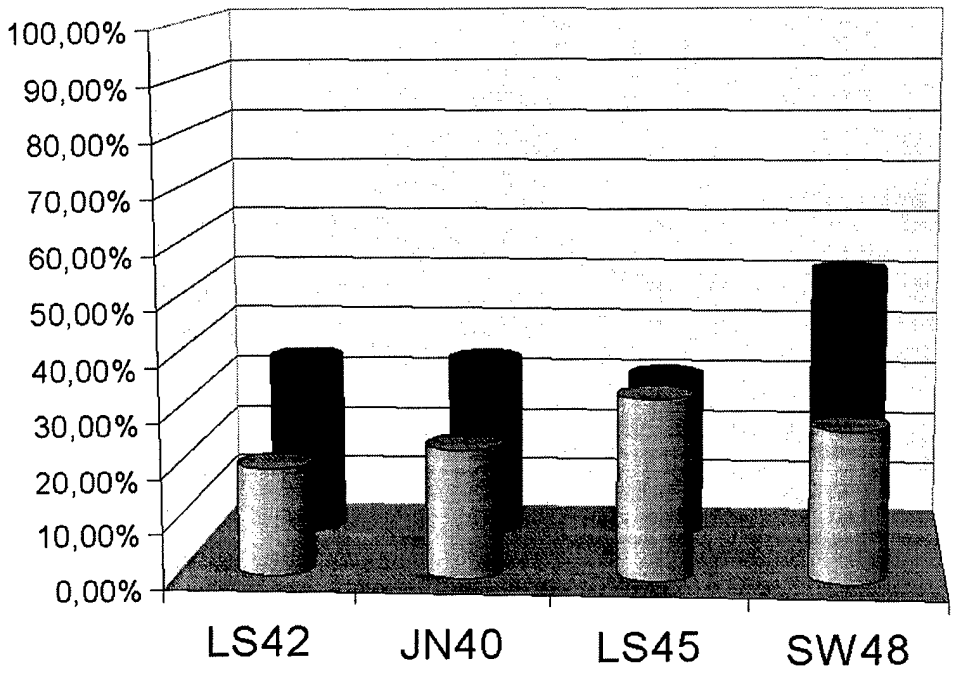

Figure 2. Expression frequency of 4 of the liver homing genes isolated from LS174T tumor cells by phage display in normal breast (light columns) and invasive breast cancers (dark columns). No significant difference in the expression frequency was observed.

We compared the pattern of expression in breast cancer and normal breast (see above) with that in normal colon, invasive colon cancer primary lesions as well as liver metastases of colon cancer (Figure 3). A highly significant increase from normal colon to invasive primary cancer to metastasis was observed and several of the genes showed 100% expression in the metastasis samples and 55% to 80% frequency in the invasive primary cancers. Normal colon samples showed 10 to 20% frequency of expression.

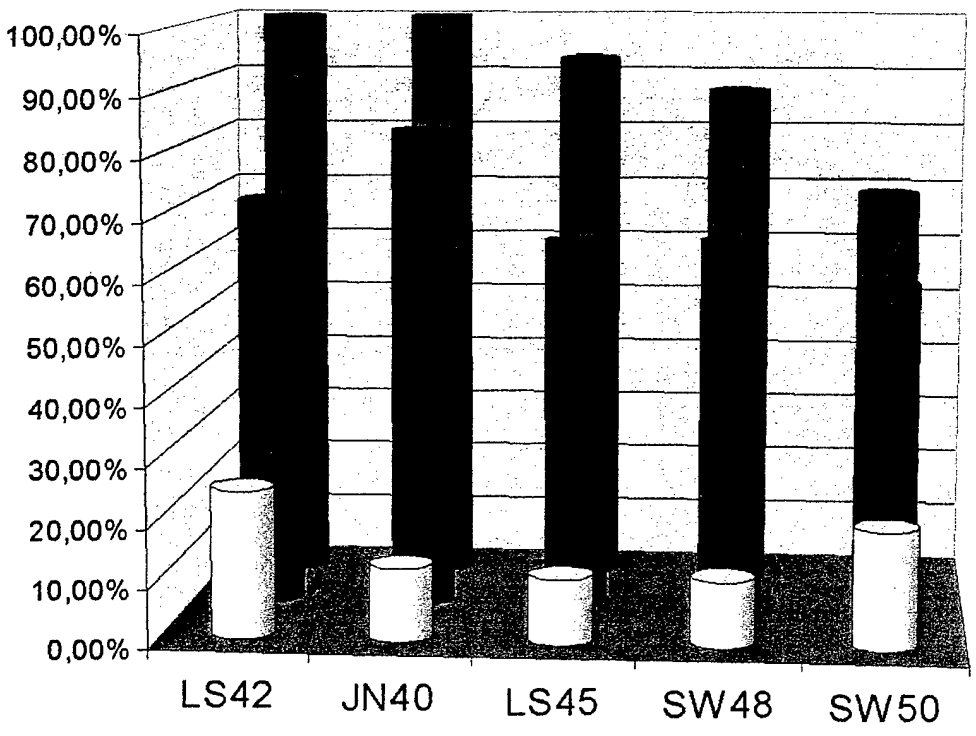

Figure 3. Expression frequency of 5 of the liver homing genes isolated from LS174T tumor cells by phage display in normal colon (light columns in the front) invasive primary colon cancers (dark columns in the middle) and liver metastases (dark columns in the back). Highly significant difference in the expression frequency was observed using chi-square analysis (Prism/Graphpad statistics program).

Methods:

Tissue samples.
Paraffin-embedded tissue samples were used for in situ hybridization and analyzed as described in the appendix materials. Tissue samples were on tissue microarrays obtained through the NCI Cooperative Breast Cancer Tissue Resource (CBCTR). This tissue array contains samples from invasive breast cancer, in situ carcinoma as well as control breast tissues from reduction mammoplasty without malignancy (CBCTR Protocol # L-0060T to the P.I. of this grant, A.W.).

These arrays contain 64 node-negative invasive breast cancers, 64 node-positive breast cancers and 64 breast cancers with distant metastasis. In addition 20 DCIS and non-malignant breast tissues are represented on the array.

Colon sample tissue microarrays were provided by Dr. Anirban Maitra from J. Hopkins University and are presented here as a positive reference.

In situ Hybridization
The method for *in situ* hybridization is described in the **appendix materials**. It is planned to post the general method of in situ hybridization on the internet at the NIH/ Cooperative Breast Cancer Tissue Resource (CBCTR) website for use by other investigators that wish to utilize tissue microarrays for the detection of the expression of genes of interest.

INTERPRETATION OF THE DATA.

We conclude that liver homing genes isolated from colon cancer cells show selective expression in colon cancer liver metastases and in primary colon cancers, however, no selective expression relative to normal tissues in breast cancer samples.

We plan to run some breast cancers with know liver metastasis to assess whether that might give a differential expression.

KEY RESEARCH ACCOMPLISHMENTS

1. We established the method to hybridize cDNAs isolated from phage display to the expressed mRNA in paraffin-embedded breast cancer samples.
2. We could not demonstrate that homing genes playing a role in liver metastasis of colon cancer have a similar role in metastatic breast cancer. However, breast cancers metastatic to the liver have not yet been evaluated.

REPORTABLE OUTCOMES

Posting for the NCI/NIH website on in situ hybridization (draft):

Henke et al.; CBCTR website NCI/NIH

CONCLUSIONS

- Phage display discovered genes can be detected in tissue samples that are paraffin-embedded.
- Liver homing genes in breast cancer may be different from those in colon cancer.

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APPENDICES

Henke et al.; CBCTR website NCI/NIH

(UNDER REVIEW FOR THE CBCTR WEBSITE AT NIH/NCI)

IN SITU HYBRIDIZATION WITH DIGOXIGENIN LABELED RNA PROBES

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Introduction

When investigating the RNA expression in tissues one of the main concerns is to distinguish between expression levels in particular cell types and structures. When using northern blot or real time PCR with heterogeneous tissue samples it is often difficult to reach exact conclusions. The in situ hybridization (ISH) is established as a method allowing demonstration of the RNA expression by using specific probes without loosing the morphological information since the tissues are preserved in there structure.

The protocol described here uses Digoxigenin labeled RNA-Probes thus avoiding any radioactive isotopes and was used in our lab for more than 1000 slides including more than 100 tissue micro arrays. Positive staining results will appear as a violet to brown staining in individual cells, mainly in the cytoplasm. Normally unstained nuclei can be observed surrounded by stained cytoplasm (**Figure 1a**). Unspecific staining can be expected especially in collagen structures and usually appears blue rather than violet. Counterstaining with e.g. Haematoxilin is not necessary and not recommended since the low staining intensity of the ISH may otherwise be difficult to evaluate. Tissue structures can still be determined even in completely negative tissue (**Figure 1b**).

When staining tissue micro arrays we recommend establishing the method of ISH and the quality of each individual probe set (antisense and sense) first on an appropriate number of full sections of according pathological and reference tissues (e.g. for a breast cancer array: two sets of 10 invasive breast cancers and 10 normal breast samples, stained in the same batch. One set used for antisense and one for sense). This is especially important since due to their limited availability and high value usually only one tissue micro array will be stained with the antisense probe and no second array for the sense probe. When advancing to tissue micro arrays with established probes we also recommend to include several slides of previously tested tissues in the same batch with the array that are known to be positive or negative for the RNA of interest. Two sets of these should be stained with the antisense and sense probe. This provides appropriate external positive and negative controls for the array.

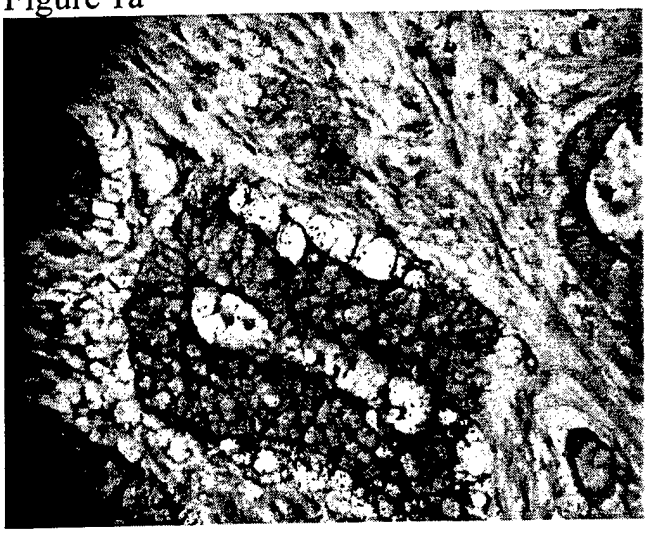
When evaluating the results of different batches of ISH high variation of staining intensity may be observed. When using tissue micro arrays each of them should be evaluated individually by first identifying the highest and lowest staining intensity on the slide.

To evaluate results we suggest using a relative scale for each array defining the highest observed staining as "+++" and the lowest as "-". Recommended coding to evaluate staining results of the individual cores is shown in **table 1**.

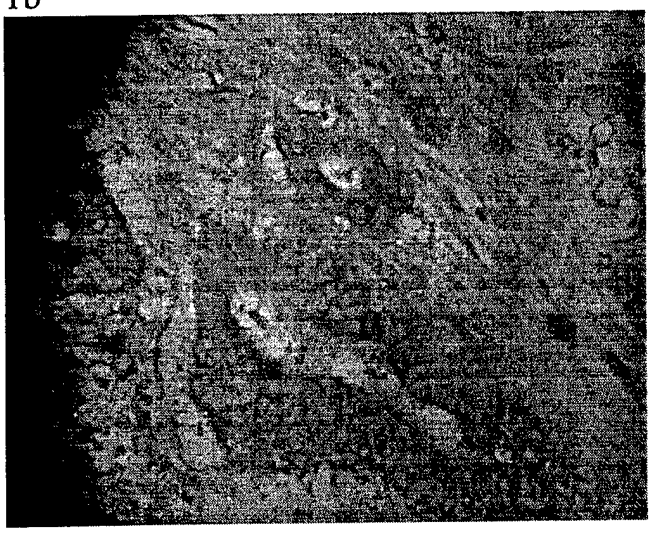
Table 1

Negative staining	-	no staining observed in this core
	+/-	slight signals but no certain positivity
Positive staining	+	certain positive staining in at least some cells
	++	medium staining in all cells or light staining in some cells and high staining in others
	+++	high staining in all cells and/or very high staining in at least 20% of the cells
N/A	x	core missing or the tissue of this core is necrotic and the RNA is degraded
	-NT	the appropriate cells (i.e. in breast arrays: cancer cells or breast epithelial cells) are not present in this core.

Figure 1a



1b



Representative staining results

1a) Colon Cancer stained with antisense RNA-probe. (400x)

1b) Sequential section stained with the according sense RNA-probe (negative control)

1. Solutions

SOLUTION (volumes are recommendations for 3-4 ISH)	NEEDED FOR
1) Digoxigenin labeled RNA-Probe / approx. 600 ng per slide (microarray)	
2) Xylene (RNA-grade)	Day 1
3) Ethanol 100% (RNA-grade)	Day 1
4) DEPC-H_2O (Diethyl Pyrocarbonate) / 8 liters for preparation of solutions - add 1ml DEPC per 1l H_2O, stir o/n then autoclave next morning	Day 1 and as stock
5) PBS 1x / 1 liter - dilute from 10x Stock with DEPC-H_2O	Day 1
6) PBS 10x / 500 ml - commercial or: - 80g NaCl, 2g KCl, 11.5g Na_2HPO_4*$7H_2O$ in 500 ml DEPC-H_2O	as stock
7) Proteinase K in PBS 1x / 200 ml per ISH - add on Day 1: 2 mg Proteinase K to 200ml PBS (final conc. 10 µg / ml)	prepare fresh on Day 1
8) SSC 2x / 1 liter - dilute from SSC 20x with DEPC-H_2O	Day 1
9) SSC 20x / 1 liter - commercial or: - 3M NaCl, 0,3 M Na-citrate, ad 1l DEPC-H_2O	as stock
10) 0,2M HCl / 1 liter - 1 Part 6N HCl, 29 Parts DEPC-H_2O	Day 1
11) TEA-HCl (0,1M Triethanolamine-HCl at ph 8,0) / 2 liters - dissolve 18,6 g TEA-HCl (RNA-grade) in 900 ml DEPC-H_2O - titrate to pH 8,0 with 10N NaOH (takes time, solution is a buffer!) - ad 1l DEPC-H_2O	Day 1
12) 0,25% Acetic anhydride in TEA-HCl / 200 ml per ISH - add on Day 1: 500 µl Acetic anhydride in 500ml in 200 ml TEA-HCl	prepare fresh on Day 1
13) Hybridization Solution / 200 µl (small sample) – 400 µl (tissue array) per slide - Sigma®, catalog number H7782 (calculate 200-400 µl/Tissue)	Day 1
14) dd-H_2O / several liters to prepare solutions and approx. 2 liters / ISH	Day 2 + Day 3
15) STE Buffer / 1 liter - 500 mM NaCl, 20 mM Tris-HCl pH 7,5, 1mM EDTA in dd-H_2O	Day 2
16) RNAse A in STE Buffer / 200 ml per ISH - ad 2g of RNAse to 200ml STE Buffer	prepare fresh on Day 2

17) SSC 2x / 2 liters - 200 ml SSC 20x, ad 2 liters dd-H_2O	Day 2
18) SSC 2x + Formamide (1:1) / 200 ml per ISH - 100 ml Formamide, 20 ml SSC 20x, 80 ml dd-H_2O	prepare fresh on Day 2
19) SSC 1x / 200 ml per ISH - 10 ml SSC 20x, 190 ml dd-H_2O	Day 2
20) SSC 0,5x / 200 ml per ISH - 5 ml SSC 20x, 195 ml dd-H_2O	Day 2
21) Buffer #1 / 3 liters - 100 mM Tris-HCl pH 7,5, 150 mM NaCl (8,77 g/l) in dd-H_2O - FILTER	Day 2 + Day 3
22) 2% Horse Serum in Buffer #1 - add 4 ml of Horse Serum to 200 ml Buffer #1	prepare fresh on Day 2
23) Anti-Digoxigenin antibody solution - dilute in Buffer #1: (calculate 500-700 µl / slide) 1:250 anti-DIG-FAB-AB-Fragments, 1:100 Horse Serum	prepare fresh on Day 2
24) Buffer #2 / 3 liters - 100 mM Tris-HCl pH 9,5, 100 mM NaCl, 50 mM $MgCl_2$ in dd-H_2O - FILTER	Day 3
25) NBT/BCIP Substrate Solution (**Light sensitive!** Wrap in aluminum-foil) - dilute in Buffer #2: (calculate 500 – 1000 µl / Tissue) 33,75µl / 10ml (3,75mg / 10ml) of NBT (nitroblue tetrazolium) 35µl / 10ml (1,75mg / 10ml) of BCIP (toluidinum salt)	prepare fresh on Day 3
26) Buffer #3 / 2 liters - 10mM Tris-HCl pH 8,0, 1mM EDTA in dd-H_2O - FILTER	Day 3
27) 0,5% Tween 20 in dd-H_2O / 200 ml per ISH	prepare fresh on Day 3
28) Sealing solution for microscopical slides	Stock

2. Material / Work environment

1) RNAse ZAP® Spray	
2) 20+ Plastic containers ("buckets") to hold the solutions during the ISH. Container-volume 200 ml	
3) Plastic slide holders for 24 slides	
4) Plastic trays ("slide chamber") to hold slides vertically during special steps like the o/n hybridization, the o/n antibody incubation and the NBT/BCIP staining	
5) Kimwipes®	
6) Parafilm®	
7) Plastic – Pasteur pipettes	
8) Vacuum system for at least 500 – 1000 ml of volume	
9) Empty drawer to develop during NBT/BCIP staining	
10) Workbench treated with RNAse ZAP®	
11) Sterile 1 liter vacuum filter system to prepare the solutions	
12) Ice on day one	
13) Water bath at 37°C	
14) Oven at 55°C and 65°C (do not use an incubator for cells or bacteria)	
15) Incubator at 42°C (do not use an incubator for cells or bacteria)	
16) Cover slips for microscopical slides	

3. Procedure

3.1. Preparations before the actual ISH

3.1.1. General considerations

- GLOVES MUST ALLWAYS BE WORN when handling the slides, any of the solutions or materials for day one or when handling the RNA-probes.
- Clean the workbench and all bottles, beakers and graded cylinders for solutions for day one with RNAse ZAP® and let them completely dry before proceeding further. Cover them with aluminum foil while drying.
- Whenever possible use sterile 15 ml or 50 ml Sarstedt® or Falcon® tubes for measuring and handling small volumes of solutions for day one. They can be considered RNAse free. Use them especially to prepare and store the hybridization solution with your RNA probe on day one.

3.1.2. Before Day 0

- Make at least 4 l of DEPC-H_2O (solution 4). If you prepare all solutions for the first time: 8 l.
- Clean the workbench with RNAse ZAP®
- Prepare solutions 5), 6), 8), 9), 10) and 11) with DEPC-H_2O
- Prepare solutions 15), 17), 21), 24) and 26) with dd-H_2O
- The three Buffer solutions must, all the others should be filtered (sterile1 liter vacuum filter system). Filter DEPC based solutions first if you want to use one filter for all. Wash filter between solutions for day one with DEPC-H_2O, later between solutions for day two and three with dd-H_2O

3.1.3. Day 0

- DO NOT MICROWAVE SLIDES, DO NOT KEEP SLIDES AT 55°C OVER NIGHT. Some other protocols recommend this preparation to remove excess paraffin. However this treatment reduces the RNA quality
- Clean the workbench (again), the plastic containers ("buckets") for day one and the slide holder with RNAse ZAP®. Place buckets and slide holder on and cover with aluminum foil. Let dry over night

3.1.4. Day 1

- Place slides in slide holder. Incubate slides in holder for 1 hour at 55°C
- Increase Oven to 65°C one hour before the first Xylene step and incubate slides for one more hour at 65°C
- Fill all **buckets for Day 1**:
  - Xylene I (Chemical hood!)
  - Xylene II (Chemical hood!)
  - Ethanol 100% I
  - Ethanol 100% II
  - DEPC-H_2O
  - PBS 1x
  - PBS 1x (later add: Proteinase K)
  - SSC 2x (DEPC-H_2O)
  - 0,2 M HCl
  - 0,1 M TEA-HCl pH 8,0
  - 0,5 ml acetic anhydride in 200 ml 0,1 M TEA-HCl pH 8,0
- Place the two PBS buckets in a 37°C water-bath
- Calculate RNA-probes and hybridization solution:
  a) needed volume of hybridization solution:
  200-400 µl/Tissue (200µl for small e.g. Cell pellets, 400 µl for Arrays)
  b) needed volume for antisense (and sense) RNA-probe:
  RNA-amount: The final RNA-concentration in the hybridization solution should be 1,5 µg RNA / 1,0 µl solution.

Example:
for 5 tissue array slides calculate 2000 µl hybridization solution and 3000 µg AS-RNA-Probe

3.1.5. Day 2

- Fill all **buckets for Day 2:**
  - SSC 2x I (dd-H_2O)
  - SSC 2x II (dd-H_2O)
  - STE Buffer
  - STE Buffer (add later: RNAse A)
  - SSC 2x + Formamide (1:1)
  - SSC 1x
  - SSC 0,5x
  - Buffer #1
  - Buffer #1 + 2% Horse serum (add later)
- Place the STE buffer buckets in 37°C water bath
- Place the SSC 2x + Formamide, the SSC 1x and the SSC 0,5x buckets in 42°C Incubator
- Take Horse Serum out of the Freezer (DO NOT heat over 37°C or microwave for thawing)

3.1.6. Day 3

- Fill all **buckets for Day 3:**
  - Buffer #1 I
  - Buffer #1 II
  - Buffer #2 I
  - Buffer #2 II
  - Buffer #3
  - 0,5% Tween
  - dd-H_2O

3.2. Day 1 – Protocol

3.2.1. De-Paraffination

- ☐ Pick up the slide holder from the 65°C oven (reset temperature to 55°C)
- ☐ Place the slide holder in Xylene I (Chemical hood!) ..... 5 min
- ☐ Xylene II (Chemical hood!) ..... 10 min
- ☐ Ethanol 100% I ..... 5 min
- ☐ Ethanol 100% II ..... 5 min
- ☐ DEPC-H_2O ..... 5 min

3.2.2. Protein-Digestion

- ☐ PBS I (37°C) ..... 5 min
- ☐ Add Proteinase K to PBS II (2 mg) while slides are in PBS I
- ☐ PBS II + Proteinase K (37°C) ..... 10 min
- ☐ PBS I (37°C) again ..... 5 min
- ☐ DEPC-H_2O (from here room temperature again) ..... 5 min

3.2.3. De-Proteinization

- ☐ 0,2 M HCl ..... 15 min
- ☐ While in HCl: add acetic anhydride to 0,1M TEA-HCl pH 8,0 bucket II
- ☐ While in HCl: pick up hybridization-solution from –20°C and RNA-probe(s) from –80°C PLACE BOTH ON ICE!
- ☐ While in HCl: prepare slide-chamber: double layer of wet filter paper, with Parafilm® on top

3.2.4. Acetylation

- ☐ 0,1M TEA-HCl pH 8,0 I ..... 5 min
- ☐ 0,1M TEA-HCl pH 8,0 II + 0,25% acetic anhydride ..... 15 min
- ☐ While in (TEA II + acet. anhy.): Mix needed volume hybridization solution with RNA-Probe. Keep ready solution on ice. Refreeze the remaining RNA-probe(s) at –80°C.

3.2.5. Hybridization

- ☐ SSC 2x (DEPC-H_2O) ..... 5 min
- ☐ Take out one slide at a time. Then do for each slide:
  - o Dry backside and front areas without tissue carefully with a Kimwipe®
  - o Place the slide vertically in the slide chamber
  - o Pipette the hybridization solution / RNA-probe mix on the tissue
- ☐ After the last Slide: seal the slide chamber with Parafilm® and place **O/N in 42°C Incubator**

3.3. Day 2 – Protocol

3.3.1. Post-Hybridization wash

- ☐ Pipette 1,0-2,0 ml of SSC 2x on each slide to dilute the hybridization solution. Use a plastic Pasteur pipette
- ☐ Suck tissues CAREFULLY almost dry with vacuum system (1 ml pipette-tip on the vacuum tube). Process one slide at a time. Do not allow the tissue to dry out completely
- ☐ Pipette 1,0-2,0 ml SSC 2x back on the tissue directly after the vacuum step
- ☐ After one complete round restart again to vacuum one slide at a time.
- ☐ Pick up the slide directly after the second vacuum step and dip it shortly in the SSC 2x I bucket. Then place it in an empty slide holder in the SSC 2x II bucket where you collect the slides
- ☐ When all slides are processed and collected in the SSC 2x II bucket, wait ..... 5 min

3.3.2. RNA-Digestion

- ☐ STE Buffer I (37°C) ..... 5 min
- ☐ While in STE Buffer I: Add 2mg RNAse A to STE Buffer II
- ☐ STE Buffer II + RNAse (37°C) ..... 10 min
- ☐ STE Buffer I (37°C) again ..... 5 min

3.3.3. Re-Fixation

- ☐ SSC 2x + Formamide (1:1) (42°C) ..... 10 min
- ☐ SSC 1x (42°C) ..... 5 min
- ☐ SSC 0,5x (42°C) 5min

3.3.4. Blocking

- ☐ Buffer #1 ..... 1 min
- ☐ Buffer #1 + 2% Horse Serum ..... 30 min
- ☐ While in Buffer #1 + HS: Prepare Antibody solution (Buffer #1 + AB + 1% Horse Serum)

3.3.5. DIG-Antibody-solution Application

- ☐ Take slides out of the blocking solution one after at a time and:
  - o Dry backside and front areas without tissue with a Kimwipe®
  - o Place slide in slide chamber
  - o Pipette Antibody solution (500+ µl / slide)
- ☐ After the last slide: seal the chamber with Parafilm®
- ☐ Place chamber in **4°C Fridge** ..... **overnight**

3.4. Day 3 – Protocol

3.4.1. Antibody washout

- Leave slides in the chamber and pipette 1000-2000µl Buffer #1 on each tissue (plastic pipette)
- After „full round" carefully vacuum liquid away and DIREKTLY re-pipette some Buffer #1
- Do a second round of vacuum. Each time a slide is finished dip it in Buffer #1 I and the place it in a slide holder in Buffer #1 II
- Once all are collected in the holder in Buffer #1 II: transfer the slides in the holder to Buffer #2 I
- Buffer #2 I ..... 5 min
- Buffer #2 II ..... 5 min
- While in Buffer #2: Prepare staining solution (NBCI/NBT in Buffer #2) (prepare in a Falcon® or Sarstedt® tube wrapped in aluminum foil)

3.4.2. Staining

- Take one slide at a time out of the Buffer #2 II, then:
  - Dry backside and front areas without tissue with a Kimwipe®
  - Place slide in the slide chamber
  - Pipette staining solution on tissues with a plastic pasteur pipette. *Note: Speed is crucial if multiple slides are processed since the first slides may already be fully stained before the last one is processed.*
- When all slides are in the chamber: Place it carefully in a drawer to develop in the dark
- Check every 10 min for staining process. If uncertain, take one of the first slides to the microscope to check and re-pipette staining solution afterwards if necessary

3.4.3. Stopping staining and cleaning

- When stained enough (usually after 20-30 min.), pick up the slides one at a time in the order they were processed, dip them in Buffer #2 and place them in a slide holder in Buffer #3
- When all slides are processed and in Buffer #3: ..... wait 5 min
- 0,5% Tween 20 (move bucket carefully to wash slides) ..... 5 min
- dd-H_2O: Place rack in bucket, go to the sink and wash under the dd-H_2O tap (slow flow) ...2 min
- Take slides out, place vertically on filter paper and let them COMPLETELY dry . at least 20 min
- Apply sealing solution on slides and cover with slip (Chemical hood)
- Wait at least 24hrs before storing slides upright in a box!

4. Further reference

Panoskaltis-Mortari, A. and R. P. Bucy (1995). "In situ hybridization with digoxigenin labeled RNA Probes: facts and artifacts." Biotechniques 18(2): 300-7